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# ENVIRONMENTAL QUALITY RESEARCH, USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINANTS Fourth Annual Report

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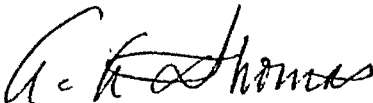
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



ANTHONY A. THOMAS, MD  
Director  
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Several species of marine and freshwater alga were used in bioassays to determine the effects of potential aquatic contamination which might result from the use of hydrazine propellants. Techniques are described for both chronic and spill types of exposure and the results of each are discussed. Compounds evaluated included hydrazine, monomethylhydrazine, and unsymmetrical dimethylhydrazine.		

## PREFACE

This is the Fourth Annual Report of work performed under the Air Force Contract AF33615-76-C-5005 and covers the period June 1, 1978 to May 31, 1979. The project is entitled "Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants." Research was conducted by the Water Resources Laboratory, School of Engineering, University of California, Irvine. The investigation was designed to expand the knowledge of toxic and biostimulatory responses of unicellular algae to hydrazine propellants and to aid Air Force personnel in assessing the environmental impact of compounds which may be released into the aquatic environment.

Contract monitor was Lt. Col. C. B. Harrah, Chief, Environmental Quality Branch of Toxic Hazards Division, AMRL, Wright Patterson Air Force Base, Dayton, Ohio. Principal investigators were Jan Scherfig, Civil and Environmental Engineering and Peter S. Dixon, Department of Ecology and Evolutionary Biology, University of California, Irvine. Project Coordinator was Mrs. Carol Justice.

The authors gratefully acknowledge the assistance of Miss Mahin Talebi for her efforts in the overall conduct of this study.

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## SUMMARY AND CONCLUSIONS

This report deals with the relative safety of hydrazine, monomethylhydrazine (MMH), and unsymmetrical dimethylhydrazine (UDMH) rocket fuels under various experimental conditions in the laboratory. Algal bioassays were conducted to determine toxic effects of these rocket fuels on several species of unicellular green algae. Safe concentrations (SC) and fifty percent effective concentrations were determined for each set of experimental conditions.

The following comparative studies were conducted to determine differences in:

1. the toxicity of hydrazine to several species of unicellular green algae which included Selenastrum capricornutum, Dunaliella tertiolecta, and Chlorella stigmatophora.
2. toxicity of UDMH to Selenastrum capricornutum, Dunaliella tertiolecta, and Chloranomala cupricola.
3. toxicity of hydrazine to pure cultures of algae with bacteria (bacterized) and without bacteria (axenic).
4. toxicity of MMH and UDMH under the spill type of exposure in which a single dose of the compound was applied and the chronic exposure in which the desired compound concentration was maintained for the duration of the experiment.

Quantitative analyses of hydrazine and monomethylhydrazine were performed in accordance with the method described by Reynolds and Thomas (1964). The method used for quantitative determination of unsymmetrical dimethylhydrazine was that described by Pinkerton et al. (1961).

Results from these studies supported the following conclusions:

1. Relative toxicity of the compound studied was, in descending order, hydrazine, MMH and UDMH the least toxic. This held true for all species of algae under all culture conditions tested.
2. Sensitivity of a particular organism was dependent upon the presence or absence of a true cell wall. Those organisms lacking the true cell wall were more sensitive to the hydrazines than those organisms which contained the carbohydrate cell wall.
3. There was no significant difference in the sensitivity of bacterized and axenic algal cultures to hydrazine.
4. Chronic exposure of the test organism to a given concentration of MMH or UDMH resulted in more severe growth inhibition than a spill exposure of the same dose.
5. All compounds studied caused some enlargement of algal cells. This was most evident with MMH and UDMH.

## INTRODUCTION

With respect to the preservation and improvement of the aquatic environment, the major emphases during the past decade have been concentrated on:

1. Increase in the effectiveness of treatment plant processes to control levels of such elements as nitrogen and phosphorus which influence the amounts entering the aquatic environment and increase the rate of eutrophication.
2. Control over discharges of industrial and commercial effluents through physical, chemical and microbial degradation.

The present research is directed towards determining possible environmental effects of various hydrazine compounds currently used or proposed for use by the U. S. Air Force as propellants for rockets. In particular, data obtained from this research will provide a sound basis for environmental impact assessment should these compounds enter the aquatic environment.

These fuels may be released once and result in a spill type of exposure or release may occur repeatedly in areas where they are constantly in use. The latter type of spill would result in a chronic exposure of the area.

Algal bioassays provide a firm basis for assessment of the impact of possible aquatic contaminants on algae over a wide range of nutrient and salinity levels. Algae are particularly significant as major primary producers in all aquatic food chains.

## OBJECTIVES

Research objectives during the past year have been directed towards determination of toxic and/or biostimulatory effects to different species of unicellular algae under different water conditions, both under a single-release basis and under chronic exposure conditions.

Quantitative bioassays were utilized to determine concentrations of the test compound necessary to cause a response of the bioassay organism. Both freshwater and marine bioassays were conducted under varying test conditions to simulate a range of aquatic ecosystems, such as oligotrophic lakes, eutrophic lakes, lakes of intermediate trophic status, estuaries and the open sea. The overall goals have been to provide information about relative safety of the compounds for environmental impact statements and determine threshold limits under which the Air Force can operate within the National Environmental Policy Act.

## SPECIFIC OBJECTIVES

1. Determination of the safe concentration (SC) for each of the compounds under the various test conditions for single exposure releases.

1. (Continued)  
The SC is defined as the highest concentration of test compound that can be administered without causing a detectable difference in standing crop at some point in time or in maximum standing crop. (Maximum standing crop is considered to have been reached when the increase in algal growth is less than five percent per day.)
2. Determination of the median effective concentration ( $EC_{50}$ ) for the compounds under the various test conditions for single exposure releases. The  $EC_{50}$  is that concentration of test compound which causes a fifty percent reduction in algal growth when compared to controls with no test compound added.
3. Determination of how much the SC and  $EC_{50}$  doses would be affected by changes in water quality and differences in algal species.
4. Comparison of the SC and  $EC_{50}$  under single exposure and chronic exposure conditions for selected compounds.
5. Comparison of the effects obtained with algae contaminated with bacteria, such as occur in natural environments, and axenic (bacteria-free) cultures to determine whether breakdown of the test compound is enhanced or retarded by the presence of the alga or its associated bacteria.

#### Workplan

The results obtained in the initial screening experiments with hydrazine were reported in the First Annual Report of this Project (1976), with the more detailed studies constituting the Second Annual Report (1977) and the Third Annual Report (1978). The present Report continues this work and a summary of results relative to hydrazine compounds, in terms of single exposure conditions, is given in Table 1.

The experiments of the present year were designed to extend the work carried out in previous years. The range of conditions used simulated the range of conditions likely to be encountered in watersheds, bays and estuaries receiving drainage from sylvan, agricultural or urban areas.

The nutrient levels for these experiments are equivalent to the following natural conditions:

Freshwater: The ranges used correspond to oligotrophic (10% SAAM nutrients), intermediate (33% SAAM nutrients) and eutrophic (100% SAAM nutrients).

Seawater: The seawater experiments were conducted over a range of salinity and nutrient levels. At a salinity of 35 ppt, the lower nutrient level (10% SAAM nutrients) is equivalent to open-sea conditions, while the higher level (33% SAAM nutrients) is equivalent to conditions encountered near to sewage outfalls or off the mouths of estuaries where nutrient-rich drainage from agriculture occurs. The experiments at lower salinities (16 ppt, 24 ppt) and the same two levels of nutrients (10% SAAM, 33% SAAM) simulate conditions found in estuaries of differing nutrient status.

TABLE 1  
HYDRAZINE COMPOUNDS TESTED AND TEST CONDITIONS FOR 1976/77  
1977/78 AND 1978/79 BIOASSAYS

COMPOUND	TEST CONDITIONS					
	Exposure	Organism	Culture	Medium	Salinity (0/00)	Nutrient Level (% SAAM)
Hydrazine	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	10
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	33
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	100
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	33
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	33
	Spill	<u>Selenastrum</u>	Axenic	Fresh	0	10
	Spill	<u>Selenastrum</u>	Axenic	Fresh	0	33
	Spill	<u>Selenastrum</u>	Axenic	Fresh	0	100
	Spill	<u>Chlorella</u>	Bacterized	Marine	24	10
	Spill	<u>Chlorella</u>	Bacterized	Marine	24	33
UDMH	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	10
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	33
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	100
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	33
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	33
	Spill	<u>Dunaliella</u>	Bacterized	Marine	35	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	35	33
	Chronic	<u>Selenastrum</u>	Bacterized	Fresh	0	33
	Spill	<u>Chloranomala</u>	Bacterized	Fresh	0	33
MMH	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	10
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	33
	Spill	<u>Selenastrum</u>	Bacterized	Fresh	0	100
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	16	33
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	24	33
	Spill	<u>Dunaliella</u>	Bacterized	Marine	35	10
	Spill	<u>Dunaliella</u>	Bacterized	Marine	35	33
	Chronic	<u>Selenastrum</u>	Bacterized	Fresh	0	33

## CONCEPTS AND METHODS

The analytical and assay procedures have been reported in detail in previous annual reports. Only modifications and new procedures developed during the present year are discussed in this annual report.

### CONCEPTS

Four key concepts are used in this work to form the basis for the conclusions regarding the effects of the different compounds in the aquatic environment.

#### Biological Growth Measures

The main concept used is the measure of biological activity. Several measures can be used including oxygen production rates, specific growth rates, and maximum biomass produced. During the early periods of this investigation extensive work was done to evaluate the applicability and methods to interpret the results obtained with each of these three parameters. Based on that work it was decided that two measures should be used to evaluate the effects of potential toxic compounds.

The first measure is maximum standing crop. The maximum standing crop is defined as the amount of algal growth (as cell numbers or total cell volume) obtained when algal growth had culminated. This is determined as the time when the increase in algal growth has stopped or decreased to less than five percent per day. Measurement of maximum standing crop may be complicated when the potentially toxic compound being tested is unstable in natural waters. This problem surfaced during the studies of hydrazine compounds. Here, the instability is such that the compound will totally decompose within a few days compared with the 10 to 15 days needed to reach maximum standing crop. Thus, even though there is a significant short-term toxic effect from hydrazines, after 10 days the net effect is sometimes biostimulatory, perhaps because the nitrogen in the hydrazines becomes available to the algae.

In order to determine the absolute and relative toxicity of the different hydrazine compounds it was therefore decided to determine the effect by the relative growth compared to a control sample after six, eight, and ten days of growth. The relative growth figures are then used to determine the toxic concentration of the hydrazine compounds.

#### Toxic Concentrations

Two different and complementary measures have been selected to quantify the toxic levels of the hydrazine compounds. The first of these is the Safe Concentration (SC). The Safe Concentration is the maximum concentration of a hydrazine which can be present without causing a statistically detectable difference in algal biomass.

The second measure used is the median effective concentration ( $EC_{50}$ ) which is that concentration which results in a 50 percent reduction in biomass at a given time when compared to the control.

## Types of Exposure

In natural circumstances to which potentially toxic materials are added, the addition may occur in either of two ways. On the one hand, addition may represent a single accidental release of some material, or, release may be occurring repetitively from a storage facility. The biological effects of these two different situations may be very different, particularly in the case of a compound which is unstable. During the present year, we have begun to examine situations equivalent to the second case--which we term "chronic" exposure. To maintain conditions of "chronic" exposure, the compound being tested is maintained in the test flasks at a standard concentration, irrespective of decomposition.

## Statistical Determination of Toxic Concentrations

The toxic concentrations have been determined on the basis of Analyses of Variance and t-tests for the specific experiments combined with interpolation between concentrations of hydrazines added. In addition, the results obtained both this year and last year are currently being refined by means of Probit Analyses and all results will be presented on this basis in the future reports as well as in the final summary report.

### METHODS

Algal bioassays were conducted in accordance with Standard Methods (American Public Health Association, 1975) and the Algal Assay Procedure: Bottle Test (United States Environmental Protection Agency, 1971) in order to determine the safe concentration (SC) and median effective concentration ( $EC_{50}$ ).

### Algal Bioassays

Modifications of the Algal Assay Procedure included the following:

1. A larger volume of medium was used (250 ml/500 ml flasks) but has been shown to have no effect with the auxiliary aeration system that was used.
2. Temperature control was  $23 \pm 3^{\circ}\text{C}$ .
3. All compounds contained in the growth medium were added in a particular order before filtration in order to prevent iron precipitation. The order of additions was sodium bicarbonate, magnesium sulfate, calcium chloride, potassium orthophosphate (mono-H), magnesium chloride, sodium nitrate and trace metals including a chelating agent.

Algal bioassays were conducted in two steps: (1) a broad screening series and (2) a fine evaluation analysis. First, a preliminary series of replicate flasks containing the algal growth medium was dosed with a broad range of concentrations (e.g. from 0.001 to 10 ppm) of the test compound. Flasks were seeded with the appropriate test organism and algal growth (both total cell number and total algal volume) was monitored with an electronic particle counter (Coulter model TA II with population accessory) until at least the control flasks without test compound reached the maximum standing crop. The maximum standing crop or maximum biomass is defined as having been achieved when the biomass increase is 5% or less per day. In this way it was possible to determine the approximate concentration where the SC and  $EC_{50}$  would be expected to occur. Then another series of flasks containing growth medium was dosed with this narrow range of concentrations of the test compound.

All flasks were seeded to an initial concentration of  $1 \times 10^6$  cells/l with the appropriate algal species. The test organism for freshwater bioassays is Selenastrum capricornutum. Initial investigations of marine waters used Dunaliella tertiolecta as the test organism but the differences in response between this alga and Selenastrum (reported in the 1978 annual report) caused us to question whether the cell type might be responsible. Selenastrum has a rigid cellulose cell wall, while this is absent in Dunaliella, the cell being bounded only by the cell membrane or plasmalemma. During the current year, other unicells have been tested on a preliminary basis. Algal growth was monitored as described previously and the SC and EC<sub>50</sub> concentrations were determined. The Standard Algal Assay Medium (SAAM) was the growth medium for freshwater bioassays and modified Burkholder's artificial seawater (ASW) with varying SAAM levels of nitrogen and phosphorus was the medium for the marine algal assays.

#### Test Compound Concentration

Test compounds were freshly prepared by serial dilution from the stock bottle immediately before being added to the bioassay flasks containing the algal cells. Five replicate flasks were prepared separately for each of the desired initial concentrations of test compound. A sample was removed from at least three of the bioassay flasks and analyzed chemically to determine whether the desired and actual concentrations were in agreement. In some cases the limit of detection for a particular compound was higher than the desired initial concentration and direct verification of the amount present was not possible. In most cases, the "desired" and "actual" initial concentrations were in very good agreement.

### RESULTS AND DISCUSSION

Investigations during the present year have concentrated on three inter-related areas. The first concerned the extreme susceptibility of the previously-used marine test organism, Dunaliella tertiolecta. The second area was concerned with the use of axenic cultures of algae for test purposes, while the third compared the effects of single exposures to potentially toxic materials with the effects when the test organisms were subjected to "chronic" exposures.

#### COMPARISON OF RESPONSES TO HYDRAZINE BETWEEN DIFFERENT SPECIES OF UNICELLULAR ALGAE

As mentioned in previous Annual Reports, differing responses of an order of magnitude have been detected between the standard freshwater test organisms, Selenastrum capricornutum, and the standard marine test organism, Dunaliella tertiolecta, when exposed to hydrazine compounds. These two standard test organisms are both green algae (Chlorophyta) although they differ in a number of respects. It is essential if environmental assessment is to be evaluated that the reasons for these different responses are determined. The differences between Selenastrum and Dunaliella are tabulated in Table 2.

TABLE 2

STRUCTURAL AND PHYSIOLOGICAL DIFFERENCES  
BETWEEN TWO STANDARD TEST ORGANISMS  
SELENASTRUM CAPRICORNUTUM AND DUNALIELLA TERTIOLECTA

	<u>Selenastrum capricornutum</u>	<u>Dunaliella tertiolecta</u>
Habitat	Freshwater	Marine
Cell wall	Present	Absent
Flagellation	Absent	Present

Of these morphological and physiological features, the presence or absence of a cell wall appeared to be the most significant point of difference between these two test organisms. Ukeles (1976) has attributed this feature as the principal cause of increased sensitivity to hydrocarbon contaminants in green algae for which a carbohydrate cell wall is lacking. For this reason, a further series of experiments was devised using a range of different test organisms with various structural and physiological characteristics.

Chloranomala cupricola is a freshwater alga with a cell wall. Pyramimonas is a marine flagellate without a cell wall, while Chlorella stigmatophora was chosen as an example of a marine unicell, non-flagellate, with a cell wall. Unfortunately, technical problems in growing these organisms under our standard test conditions prevented a full range of experiments being run. However, sufficient data were collected to give some comparisons. The data for the Safe Concentration (SC) and Fifty percent Effective Concentration (EC<sub>50</sub>) for exposure hydrazine under two different nutrient levels are given in Tables 3 and 4.

TABLE 3

SC AND EC<sub>50</sub> CONCENTRATIONS FOR HYDRAZINE WITH THREE  
ALGAL SPECIES GROWN UNDER OLIGOTROPHIC (10% SAAM) CONDITIONS

Test Alga	Day 6		Day 8		Day 10	
	SC-μl	EC <sub>50</sub> -μl/l	SC-μl/l	EC <sub>50</sub> -μl/l	SC-μl/l	EC <sub>50</sub> -μl/l
<u>S. capricornutum</u> (fresh water)	0.001	0.016	0.002	0.025	0.002	0.075
<u>C. stigmatophora</u> (marine)	0.005	0.010	0.005	0.014	0.008	0.019
<u>D. tertiolecta</u> (marine)	0.0001	0.0004	0.0001	0.0007	0.0003	0.0012

TABLE 4

SC AND EC<sub>50</sub> CONCENTRATIONS FOR HYDRAZINE WITH THREE  
ALGAL SPECIES GROWN UNDER EUTROPHIC (33% SAAM) CONDITIONS

Test Alga	Day 6		Day 8		Day 10	
	SC- $\mu\text{l/l}$	EC <sub>50</sub> - $\mu\text{l}$	SC- $\mu\text{l/l}$	EC <sub>50</sub> - $\mu\text{l/l}$	SC- $\mu\text{l/l}$	EC <sub>50</sub> - $\mu\text{l/l}$
<u>S. capricornutum</u> (freshwater)	0.001	0.013	0.019	0.035	---	---
<u>C. stigmatophora</u> (marine)	0.015	0.019	0.015	0.026	0.020	0.034
<u>D. tertiolecta</u> (Marine)	0.0001	0.0015	0.0003	0.0017	0.0008	0.0017

Using Chloranomala, only a few experiments could be run to completion because of the previously mentioned technical difficulties. However, it was possible to establish the Safe Concentrations and Fifty percent Effective Concentrations for UDMH in 33 percent SAAM medium. The results are shown in Table 5.

TABLE 5

SC AND EC<sub>50</sub> DOSES FOR UDMH WITH CHLORANOMALA IN 33% SAAM

Growth Day	SC - $\mu\text{l/l}$		EC <sub>50</sub> - $\mu\text{l/l}$	
	Cell Number	Volume	Cell Number	Volume
6	0.95	0.95	4.2	4.0
8	0.50	0.50	> 5.0	> 5.0
10	0.95	0.50	1.5	2.0

Safe concentrations determined for UDMH with Selenastrum as the test organism ranged from 0.5 - 5.0  $\mu\text{l/l}$  depending on the growth day and whether number or volume data were used. When Dunaliella was the test organism under similar nutrient conditions, SC doses ranged from 0.1 to 1.2  $\mu\text{l/l}$ . EC<sub>50</sub> doses for Selenastrum ranged from 4.7 to 14  $\mu\text{l/l}$  while EC<sub>50</sub> doses for Dunaliella ranged from 0.9 to 1.7  $\mu\text{l/l}$ . Bioassay results using UDMH indicate that Selenastrum and Chloranomala are both less sensitive to this compound than Dunaliella although the differences in sensitivity are not as great as those described earlier when hydrazine was used as the toxicant.

Although data acquisition on this aspect of the investigation is by no means complete, certain general conclusions can be made.

1. Chlorella stigmatophora is much less sensitive to hydrazine than the standard test organism Dunaliella tertiolecta, with a sensitivity not very different from that of the standard freshwater organism Selenastrum capricornutum.
2. Organisms with a rigid cell wall are more resistant to the toxicity of hydrazine compounds than those which have only a cell membrane or plasmalemma. This conclusion is of considerable biological importance. Marine and freshwater unicells devoid of a cell wall are largely overlooked by investigators because the cells are very fragile and easily disrupt under the mechanical shocks of collection. However, it is now generally agreed that such "naked" algae are very important and probably constitute as much as 50 percent of the total algal biomass.
3. The effects of hydrazine compounds in marine media are often much reduced because of the instability of the hydrazine compounds in the more complicated seawater matrix.

#### COMPARISON OF THE RESPONSE TO HYDRAZINE SHOWN BY BACTERIZED AND AXENIC CULTURES OF SELENASTRUM CAPRICORNUTUM

Many organic compounds found as contaminants in aquatic systems can be degraded by various types of organisms. In particular, the degradation properties of bacteria are probably the greatest, greater even than the single-celled algae. Under natural circumstances, single-celled algae are always associated with bacteria and those bacteria often provide the algal cell with necessary vitamins and trace organic compounds. In addition, bacteria may offer protection against toxicants when attached to the cell wall by providing a sort of buffer between the alga and the aqueous environment. Axenic bioassays were conducted with one organism (Selenastrum capricornutum) from which the associated bacteria had been removed. The differences in response to the impact of hydrazine were compared with the bioassay data obtained with bacterized cultures. No attempt was made to add to the axenic culture those species of bacteria thought to be active in the dissociation of hydrazine, nor was any attempt made to isolate and identify the bacteria normally associated with the bacterized Selenastrum cultures.

The basic question was to determine whether the breakdown of potentially toxic substances was due to the alga or to its associated bacteria.

An axenic culture of Selenastrum capricornutum was obtained by a combination of centrifugation, washing in sterile medium, and plating. This culture was maintained through a number of transfers into autoclave and sterilized SAAM medium. For axenic work, an incomplete medium is prepared and autoclave sterilized without phosphorus and trace metals. The phosphorus and trace metal solutions are prepared and autoclave-sterilized separately and added aseptically to the incomplete medium. This step prevents precipitation of these components due to the rise in pH which takes place during the autoclaving process.

Axenic bioassays were conducted in a manner somewhat different from bacterized bioassays. A large number of replicates (nine in this case) were used for controls and for each initial hydrazine concentration. Three of the replicates were counted on day 6, another three replicates on day 8, and the remaining three on day 10. Each flask was checked for axenic status by plating or liquid culture on the date the sample was taken for counting. Although samples were removed from the flasks with sterile pipets, the risk of contamination is great due to the large flask size and the awkwardness of the aeration tubing which must be removed each time with the stoppers. For these reasons it was not practical to rely on maintenance of axenic status for the large numbers of flasks necessary for the bioassays. Transfers of stock cultures were done with extreme care and this procedure required that two persons assist. Hydrazine concentrations were verified analytically at the beginning of the experiment and again on day 4. The data obtained from these experiments involving exposure of axenic Selenastrum capricornutum to hydrazine at two nutrient levels are presented in Tables 6 and 7.

TABLE 6

SC AND EC<sub>50</sub> DOSES FOR HYDRAZINE IN 10% SAAM  
WITH SELENASTRUM CAPRICORNUTUM IN AXENIC CULTURE

Growth Day	SC - $\mu\text{l/l}$		EC <sub>50</sub> - $\mu\text{l/l}$	
	Cell number	Algal Volume	Cell number	Algal Volume
6	0.004	0.004	0.008	0.008
8	0.004	0.004	0.016	0.014
10	0.004	0.004	0.020	0.018

TABLE 7

SC AND EC<sub>50</sub> DOSES FOR HYDRAZINE IN 100% SAAM  
WITH SELENASTRUM CAPRICORNUTUM IN AXENIC CULTURE

Growth Day	SC - $\mu\text{l/l}$		EC <sub>50</sub> - $\mu\text{l/l}$	
	Cell number	Algal Volume	Cell number	Algal Volume
4	0.005	0.005	0.009	0.013
6	0.005	0.005	0.024	0.021
8	0.005	0.010	0.035	0.035
10	0.005	0.010	0.89	0.089

Three sets of axenic and three of bacterized bioassays have been completed using the test organism Selenastrum capricornutum. These bioassays have been conducted at 10, 33 and 100% SAAM nutrients with hydrazine as the toxicant. Safe concentrations (SC) and fifty-percent effective concentrations ( $EC_{50}$ ) have been determined for hydrazine at the three nutrient levels for both axenic and bacterized culture conditions. The results for these bioassays are summarized in Tables 8 and 9. These results indicate that there is very little difference in the SC and  $EC_{50}$  dose between axenic and bacterized cultures. Since axenic bioassays are more time consuming and difficult to conduct, all remaining bioassays will be performed with bacterized cultures.

Furthermore, in terms of "real-world" situations, the use of bacterized cultures is far more appropriate than the use of axenic cultures. The use of axenic cultures may well have advantages in terms of "pure," scientific investigations but for the study of environmental impact, the use of bacterized cultures is imperative.

#### COMPARISON OF THE RESULTS OF SINGLE EXPOSURES AND CHRONIC EXPOSURE

The environmental impact of a toxic material depends on three variables:

1. The amount of toxic material initially applied.
2. The rate at which this toxic material is degraded in the environment.
3. The rate at which toxic material is applied continuously, in later events after the initial exposure.

With some toxic materials, there is virtually no degradation so that continuing applications simply increase the concentration of toxic material. In the case of hydrazines, which are unstable in aquatic environments, there is likely to be a difference between the effect of a single initial "spill" and the effect of continuous release. The latter we have termed "chronic exposure."

In order to determine the effects of chronic exposures, it is necessary to establish a procedure by which to maintain a constant concentration of the hydrazine compound. This requires a knowledge of decay rates, so that a constant concentration can be maintained, at least on a daily basis. The procedures are outlined in the Appendix.

Of the three hydrazines under investigation in the present study (hydrazine, UDMH, MMH), it was decided to investigate only UDMH and MMH. The basic reason for the rejection of hydrazine from this study was that the detection limits for chemical analyses of concentration are above the  $EC_{50}$  in the early days of growth. The algal cell is a more sensitive tool for the detection of hydrazine than the chemical analytical procedures. It was assumed that the  $EC_{50}$  for chronic exposure would be even less than the single-spill  $EC_{50}$  so that the dose limit required for chronic studies would be considerably below the limits of chemical detection.

TABLE 8

SAFE CONCENTRATIONS FOR HYDRAZINE (as  $\mu\text{l/l}$ ) UNDER VARYING CULTURE AND  
NUTRIENT CONDITIONS

Culture Conditions	Nutrient Level	Day 6		Day 8		Day 10	
		Number	Volume	Number	Volume	Number	Volume
Bacterized	10% SAAM	0.001	0.001	0.002	0.002	0.002	0.002
Axenic	10% SAAM	0.004	0.004	0.004	0.004	0.004	0.004
Bacterized	33% SAAM	0.001	0.001	0.010	0.010	---	---
Axenic	33% SAAM	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Bacterized	100% SAAM	0.005	0.005	---	---	---	---
Axenic	100% SAAM	0.00	0.005	0.005	0.010	0.005	0.010

TABLE 9

EC<sub>50</sub> DOSES FOR HYDRAZINE ( $\mu\text{l/l}$ ) UNDER VARYING CULTURE AND

NUTRIENT CONDITIONS

Culture Conditions	Nutrient Level	Day 6		Day 8		Day 10	
		Number	Volume	Number	Volume	Number	Volume
Bacterized	10% SAAM	0.02	0.03	0.02	0.03	0.02	0.03
Axenic	10% SAAM	0.008	0.008	0.016	0.014	0.020	0.018
Bacterized	33% SAAM	> 0.013	> 0.013	0.037	0.032	---	---
Axenic	33%	< 0.005	< 0.005	0.006	0.005	0.013	0.016
Bacterized	100% SAAM	0.041	0.025	---	---	---	---
Axenic	100% SAAM	0.024	0.021	0.035	0.035	0.089	0.089

With UDMH in 33 percent SAAM, the "no effect" or safe concentration under chronic exposure conditions was between 0.7 and 1.0  $\mu\text{l/l}$ . This compares with the previously determined SC between 1.0 and 3.0  $\mu\text{l/l}$  for single spill type of exposure. Fifty percent effective concentrations for chronic exposure were about 3  $\mu\text{l/l}$  where, by comparison, the  $\text{EC}_{50}$  dose for spill exposure ranged from 5 - 10  $\mu\text{l/l}$ . The fairly wide range of values for SC and  $\text{EC}_{50}$  doses during spill exposure is due partly to poor replication and partly to cell enlargement which tends to make SC and  $\text{EC}_{50}$  doses higher for volume data. Also, as the UDMH decomposes, the SC and  $\text{EC}_{50}$  doses change accordingly for the spill type of exposure and since UDMH decomposes more rapidly than the other hydrazine compounds studied, this range of SC and  $\text{EC}_{50}$  doses is generally larger.

With MMH in 33 percent SAAM, the SC under chronic exposure conditions was < 0.10  $\mu\text{l/l}$  compared with 0.2  $\mu\text{l/l}$  under single spill conditions. Similarly, in this medium the  $\text{EC}_{50}$  values are 0.25  $\mu\text{l/l}$  for chronic conditions and 0.5  $\mu\text{l/l}$  under single-spill conditions.

As was expected, both safe concentrations and fifty percent effective concentrations were higher for spill exposure than for chronic exposure. This difference between the spill  $\text{EC}_{50}$  dose and chronic  $\text{EC}_{50}$  dose is much more apparent for UDMH than for MMH. Since UDMH is the least toxic and also the least stable compound of the three studied, and hydrazine is the most stable and most toxic, and MMH is somewhere between, one might expect the difference between chronic and spill exposure to be rather minimal for hydrazine under these test conditions. There are no chronic exposure data for hydrazine at present, so this must be considered speculative at this time.

#### MORPHOLOGICAL CHANGES INDUCED BY EXPOSURE TO HYDRAZINE COMPOUNDS

In addition to effects of toxic substances on growth rates, alteration in morphology is another aspect of toxicity which needs to be investigated (Sorokin & Nishino, 1973). Using the Coulter Counter, it is possible to measure not only the number of cells (thereby measuring the amount of cell division) but also their mean cell volume. In all experiments it has been noticed that with exposure to UDMH and MMH there is an increase in the mean cell volume. With 4 - 5  $\mu\text{l/l}$  of UDMH, the cells are approximately twice as large as normal. Overall, the increase in algal cell size is in direct proportion to the concentration of UDMH and is observable throughout the duration of the bioassay if the UDMH concentration is maintained.

Further investigations at the fine structure level are currently under way to study the changes which occur in the algal cells when exposed to UDMH and MMH. Both scanning and transmission electron microscopy will be used in these investigations.

# APPENDIX

## MAINTENANCE OF CONSTANT CONCENTRATIONS OF HYDRAZINE COMPOUNDS IN AQUEOUS MEDIA

Hydrazine compounds are unstable in aqueous media, the rate of decay depending on the compound and the ionic concentration of the medium. The basic procedure used to maintain constant concentrations required detailed analyses at two-day intervals, with addition of the compound to make up for that amount lost by degradation.

The rates of degradation of the various hydrazine compounds are very different. With UDMH, the rate of decomposition appears to depend on the initial concentration but the degradation process is progressive. With MMH, concentrations remained relatively stable for the first 48 hours, but all flasks required additional MMH by day 4 (96 hours). On a percentage basis, the decomposition rates were greater for lower initial concentrations than for higher suggesting that there is a balance between toxicity and degradation. Based upon previous experiments to establish the Safe Concentrations and the Fifty percent Effective Concentrations, it was decided to establish conditions for constant concentrations on the following basis:

1. UDMH: 0.1  $\mu\text{l/l}$  to 5.0  $\mu\text{l/l}$  in 33 percent SAAM.
2. MMH: 0.1  $\mu\text{l/l}$  to 1.0  $\mu\text{l/l}$  in 33 percent SAAM.

Details of the additions found necessary are given in Tables 10 through 18 for UDMH and in Tables 19 through 23 for MMH.

TABLE 10

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 0.1  $\mu\text{l/l}$  UDMH  
(First Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM						
	Initial Measured	Day 2		Day 4		Day 6	
		Measured	Added	Measured	Added	Measured	Added
1	0.09	0.09	0.00	0.03	0.05	0.09	0.00
2	0.09	0.09	0.00	0.03	0.05	0.09	0.00
3	0.09	0.09	0.00	0.03	0.05	0.09	0.00
$\bar{x}$	0.09	0.09	0.00	0.03	0.05	0.09	0.00
s	0.00	0.00		0.00		0.00	

TABLE 11

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 0.5  $\mu\text{l/l}$  UDMH  
(First Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM						
	Initial Measured	Day 2		Day 4		Day 6	
		Measured	Added	Measured	Added	Measured	Added
1	0.48	0.46	0.05	0.40	0.10	0.44	0.05
2	0.52	0.48	0.05	0.40	0.10	0.48	0.05
3	0.50	0.44	0.05	0.40	0.10	0.44	0.05
$\bar{x}$	0.50	0.46	0.05	0.40	0.10	0.45	0.05
s	0.02	0.02		0.00		0.02	

TABLE 12

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 1.0  $\mu\text{l/l}$  UDMH  
(First Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM						
	Initial Measured	Day 2		Day 4		Day 6	
		Measured	Added	Measured	Added	Measured	Added
1	0.97	0.81	0.10	0.81	0.15	0.81	0.15
2	0.95	0.87	0.10	0.87	0.15	0.85	0.15
3	1.01	0.85	0.10	0.83	0.15	0.85	0.15
$\bar{x}$	0.98	0.84	0.10	0.84	0.15	0.84	0.15
s	0.03	0.03		0.03		0.02	

TABLE 13

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 2.0  $\mu\text{l/l}$  UDMH  
(First Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM						
	Initial Measured	Day 2		Day 4		Day 6	
		Measured	Added	Measured	Added	Measured	Added
1	2.00	1.83	0.15	1.79	2.0	1.67	0.25
2	2.00	1.83	0.15	1.83	2.0	1.79	0.25
3	2.00	1.87	0.15	1.83	2.0	1.75	0.25
$\bar{x}$	2.00	1.84	0.15	1.82	2.0	1.74	0.25
s	0.00	0.02		0.02		0.06	

TABLE 14

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 1.0  $\mu\text{l/l}$  UDMH  
(Second Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	0.97	0.95	0.05	0.69	0.30	0.85	0.10	0.97	0.03
2	0.99	0.95	0.05	0.63	0.30	0.93	0.10	0.97	0.03
3	0.99	0.95	0.05	0.69	0.30	0.91	0.10	0.95	0.03
$\bar{x}$	0.98	0.95	0.05	0.67	0.30	0.90	0.10	0.96	0.03
s	0.01	0.00		0.03		0.04		0.01	

TABLE 15

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 2.0  $\mu\text{L}/\text{L}$  UDMH  
(Second Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{L}/\text{L}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	2.05	1.95	0.15	1.55	0.50	1.99	0.05	1.93	0.10
2	2.03	1.83	0.15	1.55	0.50	1.99	0.05	1.95	0.10
3	2.03	1.89	0.15	1.55	0.50	1.99	0.05	1.95	0.10
$\bar{x}$	2.04	1.89	0.15	1.55	0.50	1.99	0.05	1.94	0.10
s	0.01	0.06		0.00		0.00		0.01	

TABLE 16

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 3.0  $\mu\text{L}/\text{L}$  UDMH  
(Second Bioassay)

Rep. No.	Concentration of UDMH in $\mu\text{L}/\text{L}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	2.99	2.79	0.20	2.35	0.70	2.97	0.05	2.83	0.15
2	2.95	2.75	0.20	2.23	0.70	2.89	0.05	2.79	0.15
3	2.97	2.77	0.20	2.29	0.70	2.89	0.05	2.81	0.15
$\bar{x}$	2.97	2.77	0.20	2.29	0.70	2.92	0.05	2.81	0.15
s	0.02	0.02		0.06		0.05		0.02	

TABLE 17

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 4.0  $\mu\text{l/l}$  UDMH  
(Second Bioassay)

Rep. No.	Initial Measured	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM							
		Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	4.00	3.75	0.25	3.23	0.60	3.80	0.15	3.84	0.15
2	3.96	3.70	0.25	3.43	0.60	3.88	0.15	3.84	0.15
3	4.00	3.74	0.25	3.59	0.60	3.84	0.15	3.80	0.15
$\bar{x}$	3.99	3.73	0.25	3.42	0.60	3.84	0.15	3.83	0.15
s	0.02	0.03		0.18		0.04		0.02	

TABLE 18

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS OF 5.0  $\mu\text{l/l}$  UDMH  
(Second Bioassay)

Rep. No.	Initial Measured	Concentration of UDMH in $\mu\text{l/l}$ 33% SAAM							
		Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	5.00	4.60	0.35	4.04	1.0	5.00	0.00	4.98	0.10
2	4.96	4.60	0.35	4.00	1.0	5.00	0.00	4.78	0.10
3	5.00	4.76	0.35	4.00	1.0	4.98	0.00	4.90	0.10
$\bar{x}$	4.99	4.65	0.35	4.01	1.0	4.99	0.00	4.89	0.10
s	0.02	0.09		0.02		0.01		0.10	

TABLE 19

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS FOR 0.10  $\mu\text{l/l}$  MMH

Rep. No.	Initial Measured	Concentration of MMH in $\mu\text{l/l}$ 33% SAAM							
		Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	0.10	0.10	0.00	0.03	0.08	0.03	0.05	0.05	0.08
2	0.10	0.10	0.00	0.03	0.08	0.05	0.05	0.02	0.08
3	0.10	0.10	0.00	0.03	0.08	0.04	0.05	0.03	0.08
$\bar{x}$	0.10	0.10	0.00	0.03	0.08	0.04	0.05	0.03	0.08
s	0.00	0.00		0.00		0.01		0.02	

TABLE 20

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS FOR 0.20  $\mu\text{l/l}$  MMH

Rep. No.	Initial Measured	Concentration of MMH in $\mu\text{l/l}$ 33% SAAM							
		Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	0.20	0.19	0.00	0.09	0.10	0.12	0.05	0.07	0.13
2	0.21	0.21	0.00	0.12	0.10	0.15	0.05	0.07	0.13
3	0.20	0.19	0.00	0.11	0.10	0.14	0.05	0.08	0.13
$\bar{x}$	0.20	0.20	0.00	0.11	0.10	0.14	0.05	0.06	0.13
s	0.01	0.01		0.02		0.02		0.01	

TABLE 21

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS FOR 0.30  $\mu\text{l/l}$  MMH

Rep. No.	Concentration of MMH in $\mu\text{l/l}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	0.35	0.28	---	0.21	0.13	0.28	0.08	0.25	0.15
2	0.33	0.29	---	0.21	0.13	0.26	0.08	0.14	0.15
3	0.33	0.31	---	0.21	0.13	0.25	0.08	0.14	0.15
$\bar{x}$	0.34	0.29	---	0.21	0.13	0.26	0.08	0.18	0.15
s	0.01	0.02							

TABLE 22

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS FOR 0.50  $\mu\text{l/l}$  MMH

Rep. No.	Concentration of MMH in $\mu\text{l/l}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	0.50	0.50	---	0.42	0.10	0.42	0.08	0.42	0.10
2	0.50	0.47	---	0.41	0.10	0.42	0.08	0.40	0.10
3	0.48	0.49	---	0.42	0.10	0.42	0.08	0.41	0.10
$\bar{x}$	0.49	0.49	---	0.42	0.10	0.42	0.08	0.41	0.10
s	0.01	0.02		0.01		0.00			

TABLE 23

MAINTENANCE OF CHRONIC EXPOSURE CONDITIONS FOR 1.0  $\mu\text{l/l}$  MMH

Rep. No.	Concentration of MMH in $\mu\text{l/l}$ 33% SAAM								
	Initial Measured	Day 2		Day 4		Day 6		Day 8	
		Measured	Added	Measured	Added	Measured	Added	Measured	Added
1	1.03	1.00	---	0.91	0.13	0.92	0.13	0.98	0.05
2	1.04	1.06	---	0.88	0.13	0.86	0.13	0.92	0.05
3	0.98	0.98	---	0.90	0.13	0.91	0.13	0.91	0.05
$\bar{x}$	1.02	1.01	---	0.90	0.13	0.90	0.13	0.94	0.05
s	0.03	0.04		0.02		0.03		0.04	

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